



Europäisches Patentamt
European Patent Office
Office européen des brevets

⑪ Publication number:

0 381 514
A2

⑫

EUROPEAN PATENT APPLICATION

㉑ Application number: 90301099.9

㉓ Int. Cl. 5: A61K 31/13

㉒ Date of filing: 02.02.90

㉔ Priority: 03.02.89 US 306378
07.08.89 US 390135

㉕ Date of publication of application:
08.08.90 Bulletin 90/32

㉖ Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

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㉚ Sphingosine and N-methyl-sphingosine as inhibitor of cell growth.

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㉛ A medicament for inhibiting growth of human and animal cells comprising: (1) a cell growth inhibitory amount of a one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof; and (2) a pharmaceutically acceptable carrier, diluent or excipient. A method for inhibiting growth of human and animal cells in vivo comprising contacting said cells with a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof. A medicament for inhibiting growth of human and animal cells comprising: (1) A cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof; and (2) a pharmaceutically acceptable carrier, diluent or excipient. A method of inhibiting growth of human and animal cells in vivo comprising contacting said cells with a cell growth inhibitory amount of

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one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof.

SPHINGOSINE AND N-METHYL-SPHINGOSINE AS INHIBITOR OF CELL GROWTH

This invention relates to inhibitors of cell growth due in part to inhibition of some protein kinases and also due to other as yet unexplored mechanisms, and in particular this invention relates to the recent findings by the present inventors that N-methyl-sphingosine and sphingosine are especially strong inhibitors of growth of many cells including tumor cells, cells involved in immune responses and cells involved in inflammatory responses. This finding opens the possibility of using these compounds as effective growth inhibitors of cells such as those mentioned above. Unless otherwise specified, "sphingosine" means sphingosine irrespective of D- or L- or erythro- or threo-configuration. Unless otherwise specified "N-methyl-sphingosine" means N,N-dimethyl-sphingosine or N-monomethyl-sphingosine, irrespective of D- or L- or erythro- or threo-configuration.

10 Sphingosine and sphingoid base have been implicated as inhibitors of C-kinase and EGF receptor-associated tyrosine kinase (Hannun and Bell, Science, 235, 670-674, 1987; Hannun, et al JBC, 261, 12604-12609, 1986; Kreutter, et al, JBC, 262, 1632-1637, 1987). In these studies however, the sphingosine used was from a commercial source (Sigma Chemical Company). The preparation contained various impurities, such as 3-O, 5-O methyl sphingosine and N-methyl sphingosine, since commercially available sphingosine is prepared after methanolysis of sphingomyelin or cerebroside, and these impurities are introduced during methanolysis. Furthermore, the backbone structure of sphingosine, i.e., the D-erythro configuration, is partially converted to the D-threo configuration. Therefore, based on these changes and various structures present in commercial sphingosine, the observed claim that sphingosine is an important inhibitor for C-kinase as well as EGF-receptor kinase remains ambiguous.

20 However, because of the potential growth modifying activities of sphingosine and sphingoid base and thus possible application of these compounds to modulate cell growth, this area remains an exciting area for investigation.

Accordingly, it would be desirable to be able to unambiguously identify sphingosine and sphingosine base and/or derivatives thereof, both synthetically prepared and/or prepared from naturally occurring sphingolipid, that have strong effects on a variety of cells so that concrete applications of these compounds to modulation of cell growth can be identified and used for practical purposes.

Accordingly, one object of the present invention is to provide information about the growth modifying activities of sphingosine and sphingoid base and/or derivatives thereof, obtained by using synthetic, well defined compounds or by using compounds prepared from naturally occurring sphingolipid.

30 Another object of the present invention is to provide in vitro applications of derivatives of these compounds that have strong growth inhibitory activity on a wide range of cell types.

These and other objects of the present invention have been achieved by providing a medicament for inhibiting growth of human and animal cells comprising:

(1) a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof; and
 35 (2) a pharmaceutically acceptable carrier, diluent or excipient.

The present invention also provides a medicament for inhibiting growth of human and animal tumors comprising:

(1) a cell growth inhibitory amount of a one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof; and
 40 (2) a pharmaceutically acceptable carrier, diluent or excipient.

The present invention also provides a medicament for inhibiting metastasis of human and animal tumors in vivo comprising:

(1) a cell growth inhibitory amount of a one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof; and
 45 (2) a pharmaceutically acceptable carrier, diluent or excipient.

The present invention also provides a medicament for inhibiting human and animal immune responses due at least in part to lymphocyte mitogenesis comprising:

(1) a cell growth inhibitory amount of a one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof; and
 50 (2) a pharmaceutically acceptable carrier, diluent or excipient.

The present invention also provides a medicament for inhibiting human and animal inflammatory

responses due at least in part to granulocyte and/or lymphocyte mitogenesis comprising:

(1) a cell growth inhibitory amount of a one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof; and

5 (2) a pharmaceutically acceptable carrier, diluent or excipient.

In another embodiment, the present invention provides a method for inhibiting growth of human and animal cells comprising contacting said cells with a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof.

10 The present invention also provides a method for inhibiting growth of human and animal tumors in vivo comprising contacting said tumors with a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof.

The present invention also provides a method for inhibiting metastasis of human and animal tumors in vivo comprising contacting said tumors with a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof.

15 The present invention also provides a method for inhibiting human and animal immune responses due at least in part to lymphocyte mitogenesis comprising contacting said lymphocytes with a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof.

20 The present invention also provides a method for inhibiting human and animal inflammatory responses due at least in part to granulocyte and/or lymphocyte mitogenesis comprising contacting said granulocytes and/or lymphocytes with a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof.

25 In preferred embodiments, the growth inhibitor comprises synthetically prepared N,N-dimethyl-D-erythro-sphingenine, synthetically prepared N,N-dimethyl-L-erythro-sphingenine or pharmaceutically acceptable salts thereof. Especially preferred is synthetically prepared N,N-dimethyl-D-erythro-sphingenine or pharmaceutically acceptable salts thereof.

30 In still another embodiment, the present invention provides a medicament for inhibiting growth of human and animal cells comprising:

(1) A cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof; and

35 (2) a pharmaceutically acceptable carrier, diluent or excipient.

The present invention also provides a medicament for inhibiting growth of human and animal tumors in vivo comprising:

(1) A cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl- sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof; and

40 (2) a pharmaceutically acceptable carrier, diluent or excipient.

The present invention also provides a medicament for inhibiting metastasis of human and animal tumors in vivo comprising:

(1) A cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof; and

45 (2) a pharmaceutically acceptable carrier, diluent or excipient.

The present invention also provides a medicament for inhibiting human and animal immune responses due at least in part to lymphocyte mitogenesis comprising:

(1) A cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof; and

50 (2) a pharmaceutically acceptable carrier, diluent or excipient.

55 The present invention also provides a medicament for inhibiting human and animal inflammatory responses due at least in part to granulocyte and/or lymphocyte mitogenesis comprising:

(1) A cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid

or pharmaceutically acceptable salts thereof; and

(2) a pharmaceutically acceptable carrier, diluent or excipient.

In an even further embodiment, the present invention provides a method of inhibiting growth of human and animal cells comprising contacting said cells with a cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof.

The present invention also provides a method for inhibiting growth of human and animal tumors *in vivo* comprising contacting said tumors with a cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof.

The present invention also provides a method for inhibiting metastasis of human and animal tumors *in vivo* comprising contacting said tumors with a cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl- sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof.

The present invention also provides a method for inhibiting human and animal immune responses due at least in part to lymphocyte mitogenesis comprising contacting said lymphocytes with a cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof.

The present invention also provides a method for inhibiting human and animal inflammatory responses due at least in part to granulocyte and/or lymphocyte mitogenesis comprising contacting said granulocytes and/or lymphocytes with a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof.

In preferred embodiments, the growth inhibitor comprises N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid, N-monomethyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof. Especially preferred is N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof.

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the structures of sphingosine and various synthetically prepared sphingosine derivatives.

Fig. 2 is a graph showing the inhibitory effect of sphingosine and various synthetically prepared sphingosine derivatives on the activity of C-kinase. The abscissa represents the concentration of sphingosine, synthetically prepared sphingosine derivatives, sphingosine prepared from naturally occurring sphingolipid or sulfatide in μM . The ordinate represents the relative activity of C-kinase (%). Closed circles: synthetically prepared N,N-dimethyl-D-erythro-sphingenine (N,N-DiMe-D-erythro) or sphingosine prepared from naturally occurring sphingolipid (sphingosine); Open circles: N,N-Dimethyl-L-erythro-sphingenine (N,N-DiMe-L-erythro); Closed squares: L-erythro sphingenine (L-erythro); Open squares: D-erythro-sphingenine (D-erythro); Open triangles: Sulfatide.

Fig. 3 is a graph showing the inhibitory effect of gangliosides and sulfatide on the activity of C-kinase. The abscissa represents the concentration of the gangliosides or of sulfatide in μM . The ordinate represents the relative activity of C-kinase (%). Closed circles: GT_{1b}; Open circles: GM₃; Open triangles: sulfatide. The glycolipids are abbreviated according to the system of Svennerholm (Svennerholm, L. J. Neurochem, 10, 613-623, 1963).

Fig. 4 is a graph showing the effect of N-methyl-sphingosine prepared from naturally occurring sphingolipid on growth of human colonic cancer cells HRT-18. The abscissa represents concentration of sphingosine derivatives or ceramide in μM . The ordinate represents the relative inhibition of cell growth (%). Open circles: ceramide (cer); Open triangle: N-acetylsphingosine (NAc); Closed squares: N,N-dimethyl-sphingosine (N,N-DiMe) prepared from naturally occurring sphingolipid; Closed circles: N-monomethyl-sphingosine (N-MonoMe) prepared from naturally occurring sphingolipid; Open squares: N,N-dimethyl-L-threo-sphingosine (N,N-DiMe-L-threo) prepared from naturally occurring sphingolipid.

Fig. 5 is a graph showing inhibition of human cancer cell growth in nude mice by N,N-dimethyl-sphingosine (N,N-DiMe) prepared from naturally occurring sphingolipid. The abscissa represents days. The ordinate represents tumor weight (mg). The control was phosphate buffered saline (PBS).

Fig. 6 is a graph showing the inhibition of concanavalin-A-induced mitogenesis of human peripheral blood lymphocytes by N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid. The abscissa represents the concentration, in μM , of N,N-dimethyl-sphingosine (N,N-DiMe: closed circles) prepared from naturally occurring sphingolipid, sphingosine (open triangles) or N-acetylsphingosine (NAc: open circles). The ordinate represents relative inhibition of DNA synthesis (%).

Fig. 7 is a graph showing the inhibition of IL-2-induced mitogenesis of human peripheral blood lymphocytes by N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid. The abscissa represents the concentration, in μM , of N,N-dimethyl-sphingosine (N,N-DiMe: closed circles) prepared from naturally occurring sphingolipid, sphingosine (open triangles) or N-acetylsphingosine (NAc: open circles).

The ordinate represents relative inhibition of DNA synthesis (%).

As used herein, sphingosine means sphingosine irrespective of D- or L- or erythro- or threo- configuration.

As used herein, N-methyl-sphingosine means N,N-dimethyl-sphingosine or N-monomethyl-sphingosine irrespective of D- or L-/erythro or threo configuration, unless otherwise specified.

Also, as used herein, "inhibiting" means "preventing and/or slowing".

Further, as used herein, "synthetically prepared" N-methyl-sphingosine means N-methyl-sphingosine prepared from a synthetically prepared sphingosine backbone, i.e. the structure is a synthetic product, and N-methyl-sphingosine "prepared from naturally occurring sphingolipid" means N-methyl-sphingosine prepared from sphingolipids which occur naturally, such as, for example, sphingomyelin, cerebroside, etc.

In order to clarify the ambiguity regarding whether sphingosine is an important inhibitor of protein kinases, including C-kinase and EGF-receptor kinase, and thus is a potential candidate for inhibiting cell growth, the present inventors used synthetically prepared chemically well-defined D- and L-erythro- and threo- sphingosine and their N,N-dimethyl derivatives as well as sphingosine prepared from naturally occurring sphingolipid as shown in Fig. 1 and evaluated the protein kinase inhibitory activity of these compounds. Comparison of the effect of chemically well-defined synthetically prepared stereo isomers of sphingosine and their derivatives on C-kinase activity from A413 cells (human epidermol carcinoma cells) is shown in Fig. 2, Fig. 3, and Tables I and II (See Example 2).

One of the present inventors also observed that SRC oncogene kinase activity was inhibited by sphingosine prepared from naturally occurring sphingolipid.

The following conclusions can be drawn:

1. D-erythro and L-erythro sphingenine showed a weak and similar inhibitory activity.
2. Only synthetically prepared N,N-dimethyl-D-erythro-sphingenine and sphingosine prepared from naturally occurring sphingolipid showed a strikingly strong inhibitory activity of C-kinase. Synthetically prepared N,N-dimethyl-L-erythro-sphingenine showed a weaker inhibitory activity, which was still stronger than non-substituted sphingenine having free amino groups.
3. The inhibitory activity of synthetically prepared N,N-dimethyl-D-erythro-sphingenine was higher than N-acetyl-GM₃, N-glycolyl-GM₃, and GD_{1a} gangliosides but had a similar range of inhibitory activity as the most potent inhibitory ganglioside GT_{1b}.
4. By analogy, synthetically prepared N,N-dimethyl-D-erythro-sphingenine and synthetically prepared N,N-dimethyl-L-erythro-sphingenine are expected to show similar activity on EGF receptor kinase.
5. By analogy, synthetically prepared sphingosine is expected to inhibit C-kinase.

Synthetically prepared N-methyl-sphingosines, including, N,N-dimethyl-D-erythro-sphingenine and N-methyl-L-erythro-sphingenine can be prepared by known methods comprising reductive methylation of the corresponding unmethylated sphingosine, for example D(+)-erythro-sphingenine and L(-)-erythro-sphingenine, respectively, in the presence of formaldehyde and sodium borohydride (Means and Feeney, *Biochemistry*, 7, 2192, 1968; *Chemical Modification of Proteins*, Holden-Day, Inc., San Francisco, p. 217. 1971). The resulting synthetically prepared N,N-dimethyl-sphingosine or sphingenine derivatives can be purified by TLC or HPLC. N-monomethyl derivatives were synthesized from backbone sphingenine by successive N-tert-butyloxycarbonylation (di-tert-butylcarbonate-NaHCO₃), O-acetylation (acetic anhydride-pyridine), N-methylation (methyl iodide-sodium hydride-dimethyl-formamide), and deprotection. The resulting compound was also purified by TLC and HPLC.

The synthetically prepared backbone sphingenines, D(+)-erythro-sphingenine and L(-)-erythro-sphingenine, can be prepared by various known methods, either starting from L-serine (Radunz H-E, Devant RM, Eiermann V, Liebigs Ann Chem 1103-1105, 1988; Nimkar S, Menaldino D, Merrill AH, Liotta D, *Tetrahedron Lett*, 29: 3037-3040, 1988; Herold P, *Helvetica Chimica Acta* 71: 354-362, 1988), or starting from D-glucose (Koike, K., et al *Carbohydrate Research*, 158, 113-123, 1986; Koike, K., et al, *An efficient synthesis of ceramide from D-Glucose*, *Glycoconjugate Journal*, 1, 107-109, 1984). The structure of sphingosine and of the N-methyl derivatives can be confirmed by NMR spectrometry and fast atom bombardment mass

spectrometry.

As a result of the discovery by the present inventors of the inhibitory effect on protein kinase activity of sphingosine and various sphingosine derivatives, the present invention provides a medicament for inhibiting growth of human and animal cells comprising:

- 5 (1) a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof; and
- (2) a pharmaceutically acceptable carrier, diluent or excipient.

Similarly, the present invention also provides a method for inhibiting growth of human and animal cells comprising contacting said cells with a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof.

The above-described medicament and method for inhibiting growth of human and animal cells are especially applicable to treatment of mammalian cells and to treatment of malignant or benign tumor cells.

The present invention also provides a medicament for inhibiting growth of human and animal tumors in vivo comprising:

- (1) A cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof; and
- (2) a pharmaceutically acceptable carrier, diluent or excipient.

Similarly, the present invention provides a method for inhibiting growth of human and animal tumors in vivo comprising contacting said tumors with a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof.

The above-described medicament and method for inhibiting growth of human and animal tumors in vivo are especially applicable to mammalian tumors and to malignant or benign tumors.

The present invention further provides a medicament for inhibiting metastasis of tumors in vivo comprising:

- (1) A cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof; and
- (2) a pharmaceutically acceptable carrier, diluent or excipient.

Similarly, the present invention provides a method for inhibiting metastasis of human and animal tumors in vivo comprising contacting said tumors with a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof.

The above-described medicament and method for inhibiting metastasis of tumors are especially applicable to mammalian tumors and malignant or benign tumors.

The present invention also provides a medicament for inhibiting human and animal immune responses due at least in part to lymphocyte mitogenesis comprising:

- (1) A cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof; and
- (2) a pharmaceutically acceptable carrier, diluent or excipient.

Similarly, the present invention provides a method for inhibiting human and animal immune responses due at least in part to granulocyte and/or lymphocyte mitogenesis comprising contacting said granulocytes and/or lymphocytes with a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof.

The above-described medicament and method for inhibiting human and animal immune responses due at least in part to lymphocyte mitogenesis are especially applicable to concanavalin-A-induced mitogenesis of lymphocytes and to phytohemagglutinin-A-induced mitogenesis of lymphocytes.

The above-described medicament and method for inhibiting human and animal immune responses due at least in part to lymphocyte mitogenesis are also applicable to autoimmune responses and especially to autoimmune responses due at least in part to: concanavalin-A-induced mitogenesis of lymphocytes, phytohemagglutinin A-induced mitogenesis of lymphocytes and IL-2-dependent T-cell growth.

The above-described medicaments and methods for inhibiting human and animal immune and autoimmune responses due at least in part to lymphocyte mitogenesis are especially applicable to immune responses in mammals.

The present invention also provides a medicament for inhibiting human and animal inflammatory responses due at least in part to granulocyte and/or lymphocyte mitogenesis comprising:

- (1) A cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared

sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof; and
 (2) a pharmaceutically acceptable carrier, diluent or excipient.

Similarly, the present invention provides a method for inhibiting human and animal inflammatory responses due at least in part to granulocyte and/or lymphocyte mitogenesis comprising contacting said 5 granulocytes and/or lymphocytes with a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof.

The above-described medicament and method for inhibiting human and animal inflammatory responses due at least in part to granulocyte and/or lymphocyte mitogenesis are especially applicable to inflammatory 10 responses in mammals and particularly to inflammatory responses due to concanavalin-A-induced mitogenesis of lymphocytes and phytohemagglutinin A-induced mitogenesis of lymphocytes.

For all of the above-described medicaments and methods, synthetically prepared N,N-dimethyl-D-erythro-sphingenine and N,N-dimethyl-L-erythro-sphingenine or pharmaceutically acceptable salts thereof are preferred, and synthetically prepared N,N-dimethyl-D-erythro-sphingenine and pharmaceutically acceptable 15 salts thereof are especially preferred.

In addition to the above, some of the present inventors also discovered that sphingosine prepared from naturally occurring sphingolipid and N-methyl-sphingosine prepared from naturally occurring sphingolipid has qualitatively analogous action to synthetically prepared N-methyl-sphingosine.

The sphingosine backbone can be prepared by two known methods: (1) methanolysis of natural 20 sphingolipid according to Tielfelder, H. and Klenk, E. (Die Chemie der Cerebroside und Phosphatide, J. Springer, Berlin, 1930) and modified by Gaver, R.C. and Sweeley, C.C. (J. Am. Oils Chem. Soc., 42: 294-298 1965); and (2) enzymatic hydrolysis of natural sphingolipids by glycosidase and ceramides as described by Kanfer in Hakomori, S., Handbook of Lipid Research, Volume 3, Sphingolipid Biochemistry, Kafer, J.N. and Hakamori, S. (Eds.), Plenum, New York, pages 167-247 (1983).

25 N-methyl-sphingosine can be prepared from naturally occurring sphingolipid by N-methylation in the presence of formaldehyde and sodium borohydride (Means, G.E. & Feeney, R.E., Biochemistry, 7, 2192-2201, 1968).

Suitable sources of naturally occurring sphingolipid include brain, kidney, spleen, blood cells and other organs and tissues from animals. Yeast and plant grains are also expected to be suitable sources of 30 naturally occurring sphingolipid.

The structure of sphingosine or N-methyl-sphingosine can be confirmed by mass spectrometry and ¹H-NMR spectroscopy.

Some of the present inventors compared the effect of N-methyl-sphingosine prepared from naturally occurring sphingolipid to unmethylated sphingosine. The comparisons are shown in Figs. 4 to 7. From these 35 data, the following conclusions can be drawn.

1. N,N-dimethyl-sphingosine, N-monomethyl-sphingosine and N,N-dimethyl-L-threo-sphingosine prepared from naturally occurring sphingolipid showed strong inhibitory activity on growth of human colonic cancer cells *in vitro*.

2. N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid showed a statistically 40 significant inhibitory effect on *in vivo* growth of human tumor cells in nude mice.

3. N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid showed a strong inhibitory effect on concanavalin-A-induced mitogenesis of human peripheral blood lymphocytes and of mouse splenocytes.

4. N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid showed a strong inhibitory 45 effect on IL-2-dependent mitogenesis of human peripheral blood lymphocytes and of mouse splenocytes.

Some of the present inventors have also now discovered that N-methyl-sphingosine prepared from naturally occurring sphingolipids are useful as various medicaments and for various medical treatments.

Accordingly, the present invention provides a medicament for inhibiting growth of human and animal cells comprising:

50 (1) A cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof; and

(2) a pharmaceutically acceptable carrier, diluent or excipient.

Similarly, the present invention also provides a method of inhibiting growth of human and animal cells 55 comprising contacting said cells with a cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof.

The above-described medicament and method are especially applicable to treatment of mammalian

cells and to the treatment of malignant or benign tumor cells.

The present invention also provides a medicament for inhibiting growth of human and animal tumors in vivo comprising:

- 5 (1) A cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof; and

(2) a pharmaceutically acceptable carrier, diluent or excipient.

Similarly, the present invention provides a method for inhibiting growth of human and animal tumors in vivo comprising contacting said tumors with a cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof.

The above-described medicament and method for inhibiting growth of human and animal tumors in vivo are especially applicable to mammalian tumors and to malignant and benign tumors.

The present invention also provides a medicament for inhibiting metastasis of tumors comprising:

- 15 (1) A cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof; and

(2) a pharmaceutically acceptable carrier, diluent or excipient.

Similarly, the present invention provides a method for inhibiting metastasis of human and animal tumors in vivo comprising contacting said tumors with a cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof.

The above-described medicament and method for inhibiting metastasis of tumors are especially applicable to mammalian tumors and to malignant or benign tumors.

The present invention also provides a medicament for inhibiting human and animal immune responses due at least in part to lymphocyte mitogenesis comprising:

- 25 (1) A cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof; and

(2) a pharmaceutically acceptable carrier, diluent or excipient.

Similarly, the present invention provides a method for inhibiting human and animal immune responses due at least in part to lymphocyte mitogenesis comprising contacting said lymphocytes with a cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof.

The above-described medicament and method for inhibiting human and animal immune responses due at least in part to lymphocyte mitogenesis are especially applicable to concanavalin-A-induced mitogenesis of lymphocytes and to phytohemagglutinin A-induced mitogenesis of lymphocytes.

The above-described medicament and method for inhibiting human and animal immune responses due at least in part to lymphocyte mitogenesis are also applicable to autoimmune responses and especially to autoimmune responses due to: concanavalin-A-induced mitogenesis of lymphocytes, phytohemagglutinin A-induced mitogenesis of lymphocytes and IL-2-dependent T-cell growth.

The above-described medicaments and methods for inhibiting human and animal immune responses due at least in part to lymphocyte mitogenesis are especially applicable to immune responses in mammals.

The present invention also provides a medicament for inhibiting human and animal inflammatory responses due at least in part to granulocyte and/or lymphocyte mitogenesis comprising:

- 45 (1) A cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof; and

(2) a pharmaceutically acceptable carrier, diluent or excipient.

Similarly, the present invention provides a method for inhibiting human and animal inflammatory responses due at least in part to granulocyte and/or lymphocyte mitogenesis comprising contacting said granulocytes and/or lymphocytes with a cell growth inhibitory amount of one or more growth inhibitors comprising sphingosine prepared from naturally occurring sphingolipid, N-methyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof.

The above-described medicament and method for inhibiting human animal inflammatory responses due at least in part to granulocyte and/or lymphocyte mitogenesis are especially applicable to inflammatory responses in mammals and particularly to inflammatory responses due to concanavil-A-induced

mitogenesis of lymphocytes and phytohemagglutinin A-induced mitogenesis of lymphocytes.

For all of the above-described medicaments and methods pertaining to sphingosine prepared from naturally occurring sphingolipid and N-methyl-sphingosine prepared from naturally occurring sphingolipid, N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid, N-monomethyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts thereof are preferred, and N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid and pharmaceutically acceptable salts thereof are especially preferred.

Also, for inhibiting growth of tumor cells, tumors and metastasis of tumors the medicaments and methods of the present invention are expected to be especially effective when used in combination with existing chemotherapeutic agents, such as, for example, mitomycin and 5-fluorouracil.

Suitable pharmaceutically acceptable salts of synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine, sphingosine prepared from naturally occurring sphingolipid and N-methyl-sphingosine prepared from naturally occurring sphingolipid can readily be determined by the skilled artisan.

Suitable pharmaceutically acceptable carriers, diluents or excipients for the medicaments of the present invention depend upon the particular medical use of the medicament and can readily be determined by the skilled artisan.

Suitable methods of administration of the medicaments of the present invention depend upon the particular medical application and can readily be determined by the skilled artisan.

Suitable doses of the medicaments of the present invention depend upon the particular medical application, as well as the weight of the subject, etc., and can readily be determined by the skilled artisan for example by extrapolation from in vitro data such as that shown in Fig. 2 and Table 1 for sphingosine prepared from naturally occurring sphingolipid and synthetically prepared N-methyl-sphingosine and such as that shown in Fig. 4 for N-methyl-sphingosine prepared from naturally occurring sphingolipid or from in vivo data such as that shown in Fig. 5 for N-methyl-sphingosine prepared from naturally occurring sphingolipid.

The present invention will now be described by reference to specific examples, but the invention is not to be construed as being limited thereto.

Unless otherwise specified, all percents, ratios, etc., are by weight.

30

EXAMPLE 1

35

SYNTHESIS OF SYNTHETICALLY PREPARED SPHINGOSINE DERIVATIVES

D(+)erythro-sphingenine, L(-)erythro-sphingenine, L(-)-threo-sphingenine, and L(-)-threo-sphingenine were synthesized as previously described (Koike, K., et al Carbohydrate Research, 158, 113-123, 1986; Koike, K., et al, An efficient synthesis of ceramide from D-Glucose, Glycoconjugate Journal, 1, 107-109, 1984). N,N-dimethyl derivatives were prepared by reductive methylation of erythro-sphingenine in the presence of formaldehyde and sodium borohydride (Means & Feeney, Biochemistry, 7, 2192, 1968; Chemical Modification of Proteins, Holden-Day, Inc., San Francisco, p. 217, 1971).

The synthetically prepared backbone sphingenines, D(+)erythro-sphingenine and L(-)erythro-sphingenine, can also be prepared by various known methods, either starting from L-serine (Radunz H-E, Devant RM, Eiermann V, Liebigs Ann Chem 1103-1105, 1988; Nimkar S, Senaldino D, Merrill AH, Liotta D, Tetrahedron Lett, 29: 3037-3040, 1988; Herold P, Helvetica Chimica Acta 71: 354-362, 1988), or starting from D-glucose. (Koike et al, supra).

For comparison, crude sphingosine was prepared from cerebroside by methanolysis as described originally by Tierfelder and Klenk (Die Chemie de Cerebroside und Phosphatide, Springer Verlag, Berlin, 1932).

The structures of the N,N-dimethyl-sphingosine derivatives were confirmed by NMR spectroscopy and fast atom bombardment mass spectrometry.

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EXAMPLE 2

DETERMINATION OF INHIBITION OF C-KINASE ACTIVITY BY SPHINGOSINES AND SYNTHETICALLY
PREPARED SPHINGOSINE DERIVATIVES

5

Isolation of C-kinase from A431 cells

A431 cells were grown in a mixture of DME and Ham's F-12 medium (weight ratio 1:1) supplemented with 10% fetal calf serum (FCS). Cells harvested from 50 150-cm diameter dishes were treated simultaneously for partial purification of C-kinase, by the method of Kreutter et al. (JBC, 262, 1633-1637, 1987). Briefly, cells were scraped by rubber policeman, suspended in 50 ml of 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 0.15 U/ml aprotinin, and 0.25 M sucrose, and homogenized by 50 strokes at 4°C in a Dounce homogenizer (40-ml size, Wheaton). The homogenized cells were ultracentrifuged at 100,000 xg for 60 min. The supernatant was purified on a DE52 column equilibrated with 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 0.5 mM EGTA (buffer B), and washed well with this buffer. The C-kinase activity was eluted with buffer B containing 0.1 M NaCl. The activity in this fraction was 200-500 pmol P/min/mg protein. The fraction, which was free of A-kinase and other kinases, was aliquoted and kept at -80°C.

20 Determination of Inhibitory Effects of Sphingosine and Sphingosine Derivatives on C-kinase

In view of the extremely variable results obtained in a mixed micelle system, as described by Bell and Hannun (Science 235, 670-674, 1987), the standard liposome method described by Kreutter et al. (JBC, 260 5979-5986, 1985) was slightly modified, and the effect of sphingosine and sphingosine derivatives (non-25 glycosylated and glycosylated) was studied under these conditions. In conical tubes (1.5 ml content, Sarstedt), phosphatidylserine (5 µg/tube) and 1,2-diolein (0.05 µg/tube), with or without appropriate quantity of sphingosine (sphingosine prepared from naturally occurring sphingolipid was prepared as described in Example 3), its derivatives or sulfatide, were added in organic solvent (ethanol or chloroform-methanol), and the mixture was evaporated under an N₂ stream. The lipid mixture was sonicated in 30 µl of 20 mM Tris-30 HCl (pH 7.5) for 30 min. The liposomes in the tube were supplemented with the reaction mixture, consisting of 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 400 µM EDTA, 50 µM EGTA, 500 µM CaCl₂, 200 µg/ml Histone III-S, and 20 µM gamma-[³²P]-ATP (2 x 10⁶ cpm); final volume was 90 µl.

The reaction was initiated by addition of 10 µl of C-kinase fraction (containing 1-2 µg protein) prepared as described above, and the reaction mixture was incubated for 10 min at 30°C. The reaction was terminated by addition of 1 ml of 25% TCA with 200 µl of 1% BSA in 1 mM ATP solution (pH 7.5). The precipitate was centrifuged, washed twice with 1 ml of 25% TCA, dissolved in 1 ml of 1 N NaOH containing 0.1% deoxycholate with slight heating (80°C for 10 min), and counted in a scintillation counter. The value without phosphatidylserine, 1,2-diolein, or Ca²⁺ were used as a control. The effects of sphingosine and sphingosine derivatives and are shown in Fig. 2 and Fig. 3 and are summarized in Table I and Table II 40 below.

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TABLE I

5 Inhibitory effect of sphingosine and various
sphingosine derivatives on C-kinase activity

10	Added sphingolipid	Inhibition of C-kinase (concentration in μ M yielding 50% inhibition)
<hr/>		
15	A. <u>Sphingosines and non-glycosylated derivatives</u>	
20	Ceramide	no inhibition
25	Sphingosine (D-erythro, SigmaSM)	25
30	Sphingosine (D-erythro, SigmaCer)	25
	Sphingosine (D-erythro)	55
	Sphingosine (L-erythro)	55
	Sphingenine (L-threo)	30
	Sphinganine (L-threo)	50
35	Sphingenine (DL-erythro, Sigma)	no data
40	N-acetylsphingosine	no inhibition
45	D-erythro-N,N-dimethyl- sphingosine	5-10
	L-erythro-N,N-dimethyl- sphingosine	25
50	Sphingosine prepared from naturally occurring sphingolipid	5-10

TABLE I (CONTINUED)

5	Added sphingolipid	Inhibition of C-kinase (concentration in μM yielding 50% inhibition)
10	B. <u>Glycosylated derivatives</u>	
15	Lactosylsphingosine	no inhibition
	GM ₃ (N-acetyl)	20
	GM ₃ (N-glycolyl)	25
	GD _{1a}	20
	GT _{1b}	10

20

TABLE II

25	Inhibition of C-kinase activity by sphingosines			
	Lipid Added	Amount (μM)	C-kinase ^{32}P incorporation	%
30	Phosphatidyl-serine, diolein, Ca^{+2}	*	39620 \pm 4156	100 \pm 10
	D-erythro-sphingenine	50 μM	18754 \pm 2184	47 \pm 6
	L-erythro-sphingenine	50 μM	18549 \pm 2760	47 \pm 7
35	N,N-dimethyl-D-erythro-sphingenine	2 μM	34206 \pm 1220	86 \pm 3
	I_{50} = 10 μM	10 μM	20563 \pm 1173	52 \pm 3
		25 μM	15214 \pm 377	38 \pm 1
		50 μM	5254	13
40	N,N-dimethyl-L-erythro-sphingenine	2 μM	37354 \pm 1420	95 \pm 4
	I_{50} = 25 μM	10 μM	25583 \pm 1931	65 \pm 5
		25 μM	21743 \pm 577	55 \pm 2
		50 μM	11020	28

* See text

From the data shown in Table I and Table II and the results in Fig. 2 and Fig. 3, the following conclusions can be drawn:

- 45 1. Synthetically prepared D-erythro and L-erythro sphingenine showed a weak and similar inhibitory activity.
2. Only synthetically prepared N,N-dimethyl-D-erythro-sphingenine and sphingosine prepared from naturally occurring sphingolipid showed a strikingly strong inhibitory activity of C-kinase. N,N-dimethyl-L-erythro-sphingenine showed a weaker inhibitory activity, which was still stronger than non-substituted sphingenine having free amino groups.
- 50 3. The inhibitory activity of synthetically prepared N,N-dimethyl-D-erythro-sphingenine was higher than N-acetyl-GM₃, N-glycolyl-GM₃, and GD_{1a} gangliosides but had a similar range of inhibitory activity as the most potent inhibitory ganglioside GT_{1b}.

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EXAMPLE 3

SYNTHESIS OF SPHINGOSINE DERIVATIVES PREPARED FROM NATURALLY OCCURRING SPHINGOLIPID

Sphingosine was prepared after methanolysis of brain sphingolipid fraction according to methods originally described by Thierfelder H. & Klenk E. (Die Chemie der Cerebroside und Phosphatide, J. Springer, Berlin, 1930) and modified by Gaver R.C. and Sweeley C.C. (J Am Oil Chem Soc 42: 294-298, 1965). Briefly, crude sphingolipid was dissolved in methanol containing 0.1 M HCl (prepared from aqueous concentrated HCl), and heated under reflux overnight. The methanolic HCl solution was shaken out with petroleum ether (hexane) to eliminate fatty acids. The lower phase was neutralized, pH adjusted to 10 with NaOH, followed by shaking out with ethylether. The ethylether layer (upper layer) containing sphingosine base was evaporated to dryness and further purified on HPLC, and the peak corresponding to D-erythrosphinganine was separated.

Sphingosine can also be prepared by hydrolysis of sphingolipids with glycosidases and phosphodiesterases followed by treatment with ceramidase (Kanfer J.N., pp. 167-247 in: Kanfer J.N. & Hakomori S. (eds.), Sphingolipid Biochemistry (Handbook of Lipid Research, Vol. 3, Plenum, NY, 1983). Briefly, cerebroside can be treated with β -galactosidase or β -glucosidase in the presence of Triton X-100. Sphingomyelin can be treated with phosphodiesterase in the presence of Triton X-100. Thus, the liberated ceramide can then be hydrolyzed with ceramidase to release sphingosine. More recently, a ceramidase which acts directly on sphingolipids has been found in Nocardia sp. (Hirabayashi Y., et al., J Biochem (Tokyo) 103: 1-4, 1988).

²⁵ N,N-dimethyl and N-monomethyl-sphingosine used for Examples 4 to 7 were prepared exclusively from sphingosine preparations derived from brain sphingolipids followed by N-methylation in the presence of formaldehyde and sodium borohydride (Means, G.E. & Feeney, R.E., Biochemistry, 7, 2192-2201, 1968) and its structure was identified by mass spectrometry and ¹H-NMR spectroscopy.

EXAMPLE 4

30

DETERMINATION OF INHIBITION OF HUMAN COLONIC CANCER CELL GROWTH BY SPHINGOSINES AND SPHINGOSINE DERIVATIVES PREPARED FROM NATURALLY OCCURRING SPHINGOLIPID

35 The effect of N,N-dimethyl-sphingosine, N-monomethyl-sphingosine and N,N-dimethyl-L-threo-sphingosine (all prepared from naturally occurring sphingoglycolipids as described in Example 3) on growth of human colonic cancer cells HRT-18 (obtained from ATCC) was tested in Dulbecco's modified Eagle's medium as follows.

40 HRT-18 cells were seeded in plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (5×10^5 cells per each 3 cm plate). After the cells were cultured overnight, the medium was replaced with the same media containing various concentrations of N,N-dimethyl- or N-monomethyl sphingosine, N-acetylsphingosine, and ceramide as shown in Fig. 4. Cultures were continued for 3 days, and cell numbers were counted. The percent inhibition of cell numbers in the presence of reagents was calculated as percent of control dishes, which contained no sphingosine derivatives.

45 The results are shown in Fig. 4.

In Fig. 4 the abscissa represents concentration of sphingosine or ceramide in μ M. The ordinate represents the relative inhibition of cell growth (%). Open circles: ceramide (Cer); Open triangles: N-acetylsphingosine (NAc); Closed squares: N,N-Dimethyl-sphingosine (N,N-DiMe) prepared from naturally occurring sphingolipid; Closed circles: N-monomethyl-sphingosine (N-MonoMe) prepared from naturally occurring sphingolipid; Open squares: N,N-dimethyl-L-threo-sphingosine (N,N-DiMe-L-threo) prepared from naturally occurring sphingolipid.

The results show that N,N-dimethyl-sphingosine as well as N-monomethyl-sphingosine and N,N-dimethyl-L-threo-sphingosine significantly inhibited at 12.5 μ M concentration, whereas ceramide or N-acetylsphingosine showed minimum inhibition even at 150 μ M concentration.

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EXAMPLE 5

DETERMINATION OF INHIBITION OF HUMAN COLONIC CANCER CELL GROWTH IN NUDE MICE BY SPHINGOSINES AND N,N-DIMETHYLSPHINGOSINE PREPARED FROM NATURALLY OCCURRING SPHINGOLIPID

5 The effect of N,N-dimethyl-sphingosine (prepared from naturally occurring sphingolipid as described in Example 3) on *in vivo* growth of HRT18 tumor cells in nude mice was determined as follows.

10 1×10^6 HRT-18 human colonic cancer cells were inoculated into nude mice at day 0 by known methods. N,N-dimethylsphingosine emulsified in phosphate buffered saline (PBS) (44 μ g/200 μ l) was injected at day 7, 11, and 15. Tumor growth weight in mg was estimated by the size of the tumors. Each group consisted of ten animals.

The results are shown in Fig. 5.

In Fig. 5, the abscissa represents days and the ordinate represents tumor weight (mg). The open circles represent the control, PBS alone. The closed circles represent N,N-dimethyl-sphingosine (N,N-DiMe).

15 The results show that *in vivo* growth of HRT18 tumor cells in nude mice was inhibited by N,N-dimethyl-sphingosine. The inhibition of growth was more than 50%, and statistical treatment showed that the inhibition was significant ($p > 0.005$).

20

EXAMPLE 6

DETERMINATION OF INHIBITION OF CONCANAVALIN-A-INDUCED MITOGENESIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND MOUSE SPLENOCYTES BY N,N-DIMETHYL-SPHINGOSINE PREPARED FROM NATURALLY OCCURRING SPHINGOLIPID

30 The effect of N,N-dimethyl-sphingosine (prepared from naturally occurring sphingolipid as described in Example 3) on concanavalin-A (con-A) -induced mitogenesis was tested using human peripheral blood lymphocytes and mouse splenocytes.

35 Peripheral blood lymphocytes isolated from human blood by known methods were cultured in RPMI containing 2 μ g con-A/ml for 18 hours in multiple wells. Subsequently, various concentrations of N,N-dimethylsphingosine, sphingosine, or N-acetylsphingosine as shown in Fig. 6 were added to the culture media and cultured continuously for 5 hours, followed by addition of 3 H-thymidine. The thymidine uptake over a 5-hour period was determined. The % inhibition of mitogenesis was calculated based on control value (without addition of any inhibitor).

Inhibition of con-A-induced mitogenesis of mouse splenocytes was determined in an analogous manner.

The results for peripheral blood lymphocytes are shown in Fig. 6.

40 In Fig. 6, the abscissa represents the concentration, in μ M, of N,N-dimethyl-sphingosine (N,N-DiMe: closed circles) prepared from naturally occurring sphingolipid, sphingosine (open triangles) or N-acetyl-sphingosine (NAC: open circles). The ordinate represents relative inhibition of DNA synthesis (%).

45 The data in Fig. 6 show that con-A-dependent growth stimulation was greatly inhibited in the presence of N,N-dimethyl-sphingosine but not in the presence of unsubstituted sphingosine or N-acetylsphingosine.

Similar results were obtained using mouse splenocytes.

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EXAMPLE 7

DETERMINATION OF INHIBITION OF IL-2-DEPENDENT MITOGENESIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND MOUSE SPLENOCYTES BY N,N-DIMETHYL-SPHINGOSINE PREPARED FROM NATURALLY OCCURRING SPHINGOLIPID

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The effect of N,N-dimethyl-sphingosine (prepared from naturally occurring sphingolipid as described in Example 3) on IL-2-dependent mitogenesis was tested using human peripheral blood lymphocytes and

mouse splenocytes.

Human peripheral blood lymphocytes isolated from human blood by known methods were cultured in RPMI containing 0.5 U/ml IL-2. Subsequently, the medium was replaced with media containing various concentrations of N,N-dimethylsphingosine, sphingosine, and N-acetylsphingosine as shown in Fig. 7, followed by addition of ^3H -thymidine. Mitogenesis was measured by incorporation of ^3H -thymidine into DNA over a 5-hour period. Percent inhibition as compared to the control value (without addition of any inhibitor) was determined.

Inhibition of IL-2-induced mitogenesis of mouse splenocytes was determined in an analogous manner.

The results for peripheral blood lymphocytes are shown in Fig. 7.

In Fig. 7, the abscissa represents the concentration, in μM , of N,N-dimethyl-sphingosine (N,N-DiMe: closed circles) prepared from naturally occurring sphingolipid, sphingosine (open triangles) or N-acetyl-sphingosine (NAc: open circles). The ordinate represents relative inhibition of DNA synthesis (%).

The data in Fig. 7 show that mitogenesis was invariably inhibited in the presence of N,N-dimethyl-sphingosine but less so in the presence of unsubstituted sphingosine or N-acetylsphingosine.

Similar results were obtained using mouse splenocytes.

From the results of Examples 4 to 7, the following conclusions can be drawn:

1. N,N-dimethyl-sphingosine, N-monomethyl-sphingosine and N,N-dimethyl-L-threo-sphingosine prepared from naturally occurring sphingolipid showed strong inhibitory activity on growth of human colonic cancer cells *in vitro*.

2. N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid showed a statistically significant inhibitory effect on *in vivo* growth of human tumor cells in nude mice.

3. N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid showed a strong inhibitory effect on concanavalin-A-induced mitogenesis of human peripheral blood lymphocytes and of mouse splenocytes.

4. N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid showed a strong inhibitory effect on IL-2-dependent mitogenesis of human peripheral blood lymphocytes and of mouse splenocytes.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

Claims

35 1. The use of one or more cell growth inhibitors selected from synthetically prepared sphingosine, sphingosine prepared from naturally occurring sphingolipid synthetically prepared N-methyl sphingosine, N-methyl-sphingosine prepared from naturally occurring sphingolipid and pharmaceutically acceptable salts thereof in surgery therapy or diagnosis.

40 2. A use as claimed in claim 1 wherein the cell growth inhibitor comprises N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid, or N-monomethyl-sphingosine prepared from naturally occurring sphingolipid or synthetically prepared N,N-dimethyl-D-erythro-sphingenine, synthetically prepared N,N-dimethyl-L-erythro-sphingenine, or pharmaceutically acceptable salts

45 3. A use as claimed in claim 2 wherein the cell growth inhibitor comprises synthetically prepared N,N-dimethyl-D-erythro-sphingenine, or N,N-dimethyl-sphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts.

4. A use as claimed in any one of claims 1 to 3 in inhibiting mammalian cell growth.

5. A use as claimed in any one claims 1 to 3 practiced on malignant or benign tumor cells.

6. The use of one or more cell growth inhibitors selected from synthetically prepared sphingosine, sphingosine prepared from naturally occurring sphingolipid, synthetically prepared N-methyl sphingosine N-methyl-sphingosine prepared from naturally occurring sphingolipid and pharmaceutically acceptable salts thereof in the manufacture of a medicament for inhibiting growth of human, mammalian or other animal tumors.

7. The use of one or more cell growth inhibitors selected from synthetically prepared sphingosine, sphingosine prepared from naturally occurring sphingolipid, synthetically prepared N-methyl sphingosine N-methyl-sphingosine prepared from naturally occurring sphingolipid and pharmaceutically acceptable salts thereof in the manufacture of a medicament for inhibiting metastasis of human, mammalian or other animal tumors.

8. The use of one or more cell growth inhibitors selected from synthetically prepared sphingosine,

sphingosine prepared from naturally occurring sphingolipid, synthetically prepared N-methyl sphingosine, N-methyl-sphingosine prepared from naturally occurring sphingolipid and pharmaceutically acceptable salts thereof in the manufacture of a medicament for inhibiting human and animal, preferably mammalian, immune responses due at least in part to lymphocyte mitogenesis.

- 5 9. A use as claims in claim 8 for the manufacture of a medicament for inhibiting an autoimmune response.
- 10 10. A use as claimed in claim 9 for the manufacture of a medicament for inhibiting an autoimmune response due at least in part to IL-2-dependent T-cell growth.
- 11 11. A use as claimed in claim 8 or claim 9 for the manufacture of a medicament for inhibiting an immune response due at last in part to concanavalin-A induced mitogenesis of lymphocytes.
- 12 12. A use as claimed in claim 8 or claim 9 for the manufacture of a medicament for inhibiting an immune response due at least in part to phytohemagglutinin-A induced mitogenesis of lymphocytes.
- 13 13. The use of one or more cell growth inhibitors selected from synthetically prepared sphingosine, sphingosine prepared from naturally occurring sphingolipid, synthetically prepared N-methyl sphingosine N-methyl-sphingosine prepared from naturally occurring sphingolipid and pharmaceutically acceptable salts thereof for the manufacture of a medicament for inhibiting human and animal inflammatory response due at least in part to granulocyte or lymphocyte mitogenesis.
- 14 14. A use as claimed in claim 13 for the manufacture of a medicament for inhibiting mammalian inflammatory response.
- 20 15. A use as claimed in claim 13 wherein the inflammatory response, is due at least in part to concanavalin-A induced mitogenesis of lymphocytes or phytohemagglutinin-A induced mitogenesis of lymphocytes.
- 25 16. A use as claimed in any one of claims 6 to 15 wherein the cell growth inhibitors comprise N,N-dimethylsphingosine prepared from naturally occurring sphingolipid, or N-monomethyl-sphingosine prepared from naturally occurring sphingolipid or synthetically prepared N,N-dimethyl-D-erythro-sphingenine, synthetically prepared N,N-dimethyl-L-erythro-sphingenine, or pharmaceutically acceptable salts.
- 30 17. A use as claimed in claim 16 wherein the cell growth inhibitor comprises synthetically prepared N,N-dimethyl-D-erythro-sphingenine, or N,N-dimethylsphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts.
- 35 18. A method of inhibiting growth of human and animal cells comprising contacting the cells with one or more cell growth inhibitors selected from synthetically prepared sphingosine, sphingosine prepared from naturally occurring sphingolipid, synthetically prepared N-methyl sphingosine, N-methyl-sphingosine prepared from naturally occurring sphingolipid and pharmaceutically acceptable salts thereof.
- 36 19. A method as claimed in claim 18 wherein the cell growth inhibitor comprises N,N-dimethylsphingosine prepared from naturally occurring sphingolipid, or N-monomethyl-sphingosine prepared from naturally occurring sphingolipid or synthetically prepared N,N-dimethyl-D-erythro-sphingenine, synthetically prepared N,N-dimethyl-L-erythro-sphingenine, or pharmaceutically acceptable salts
- 40 20. A method as claimed in claim 19 wherein the cell growth inhibitor comprises synthetically prepared N,N-dimethyl-D-erythro-sphingenine, or N,N-dimethylsphingosine prepared from naturally occurring sphingolipid or pharmaceutically acceptable salts.
- 41 21. A method as claimed in any one of claims 18, 19 or 20 wherein the cells are mammalian cells.
- 42 22. A method as claimed in any one of claims 18, 19 or 20 wherein the cells are malignant or benign tumor cells.
- 43 23. A method for inhibiting animal, human or other mammalian inflammatory responses due at least in part to granulocyte and/or lymphocyte mitogenesis comprising contacting said granulocytes and/or lymphocytes with a cell growth inhibitory amount of one or more growth inhibitors comprising synthetically prepared sphingosine, synthetically prepared N-methyl-sphingosine or pharmaceutically acceptable salts thereof.

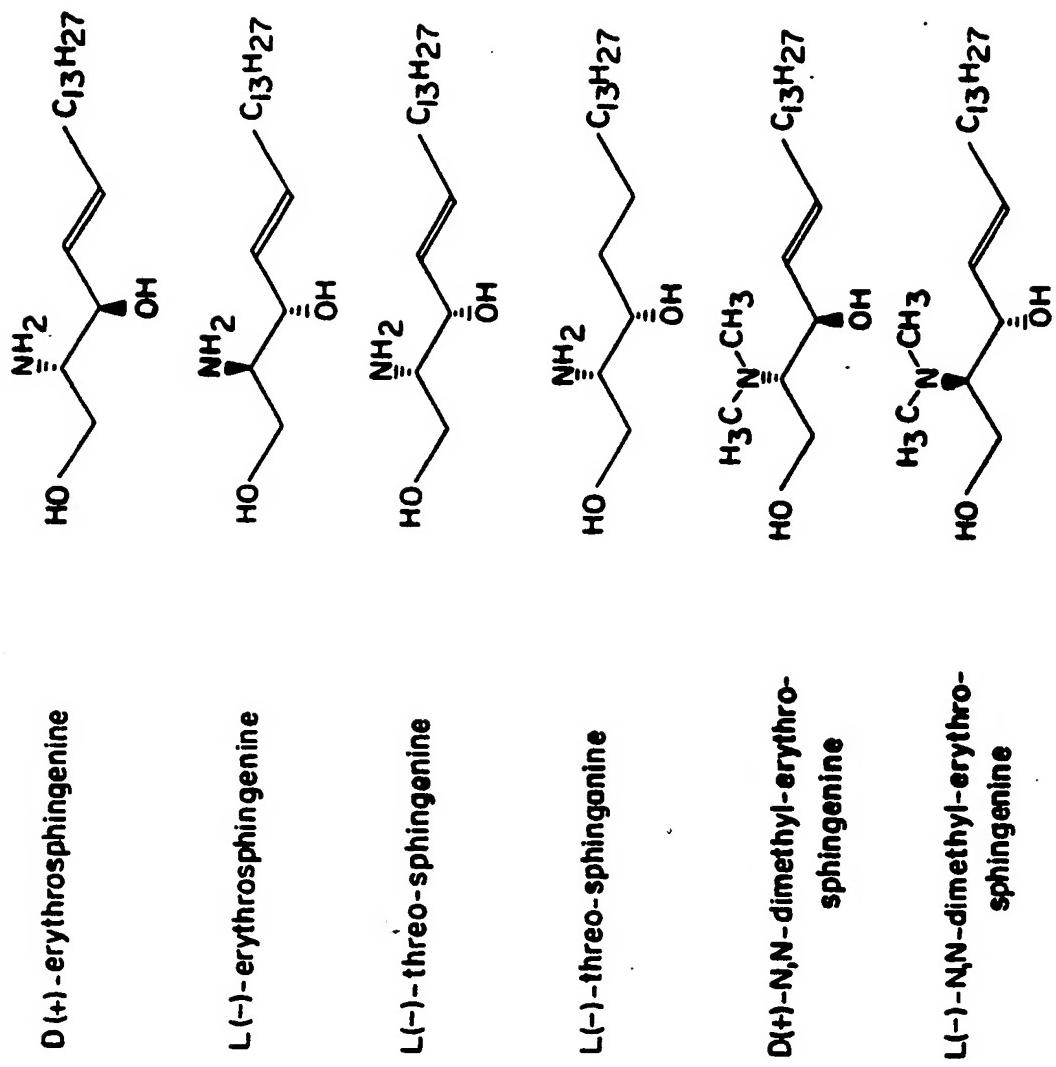


FIG. 1

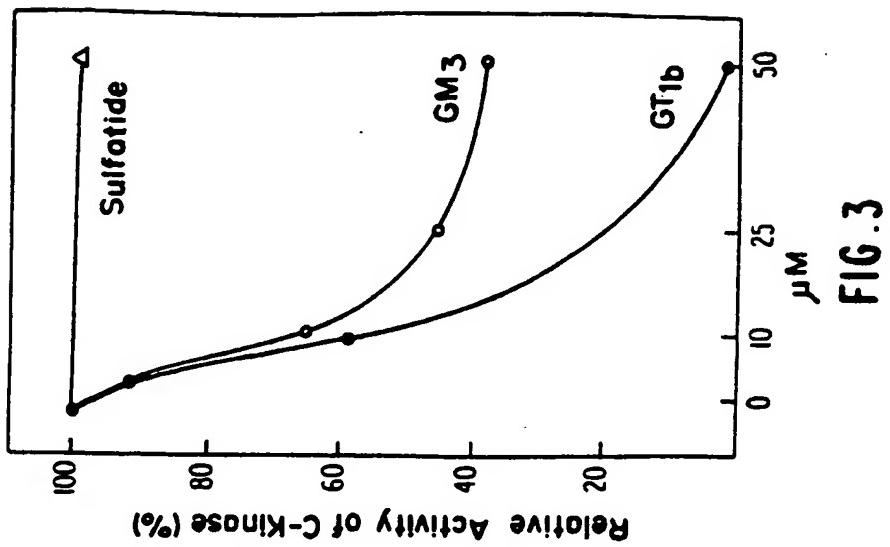


FIG. 3

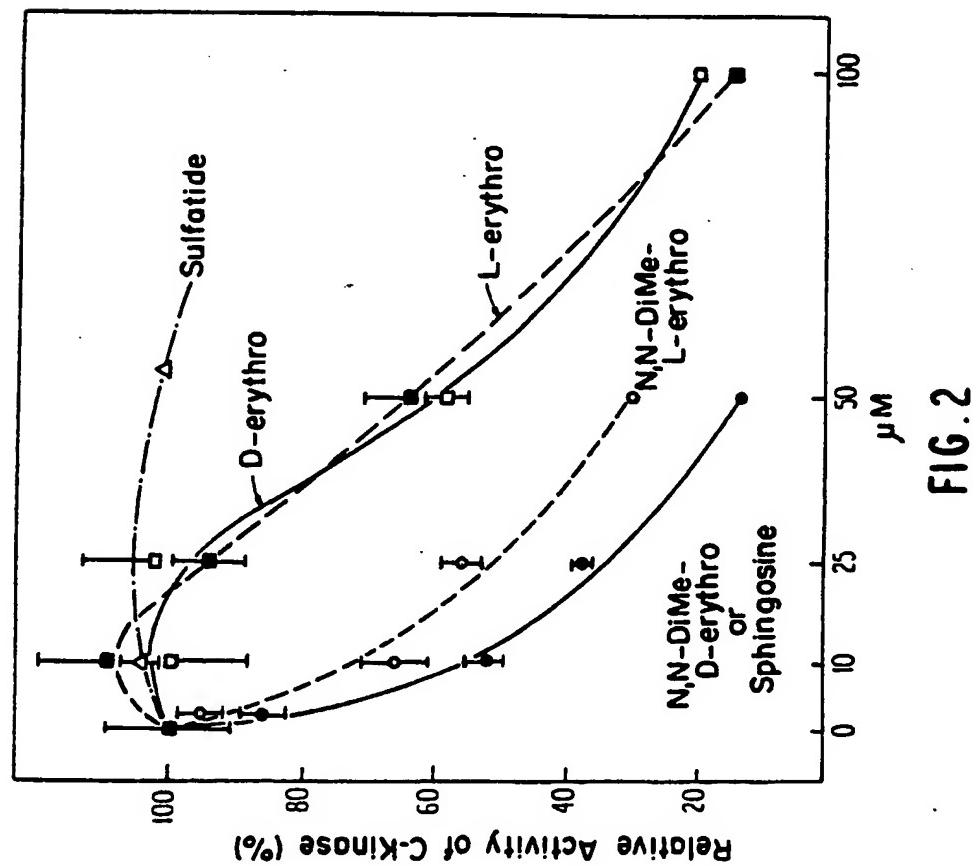


FIG. 2

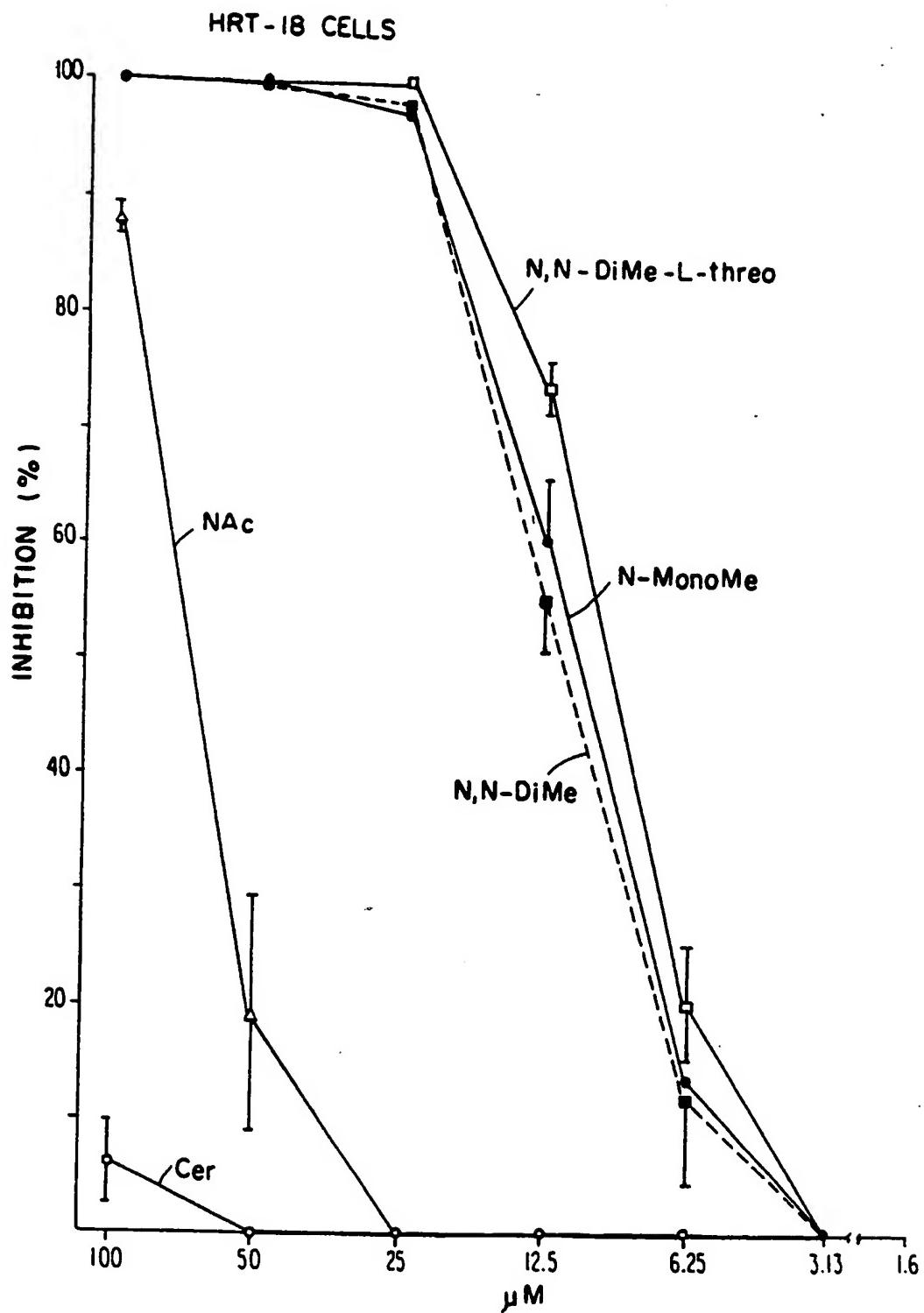


FIG.4

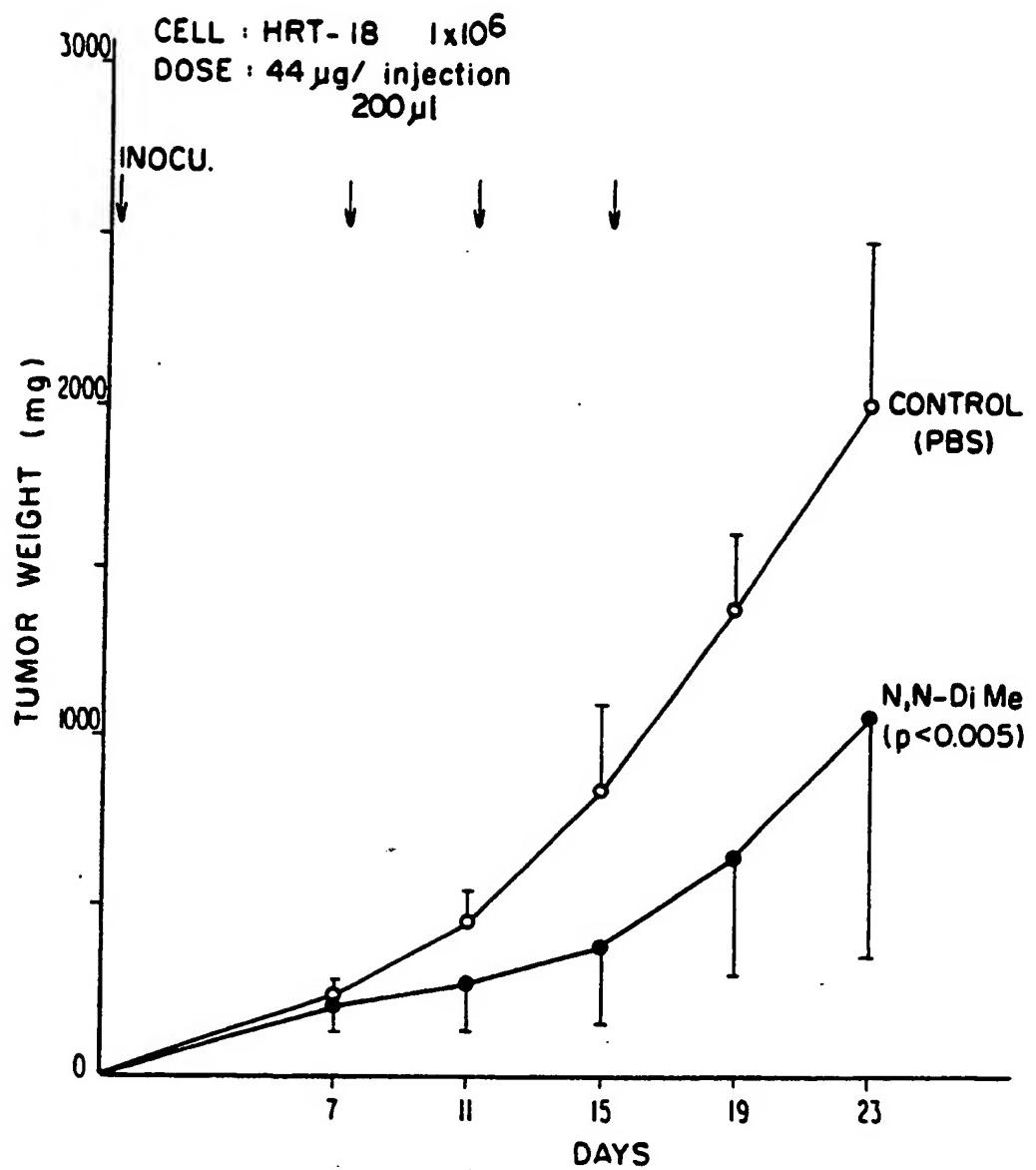


FIG. 5

FIG. 6

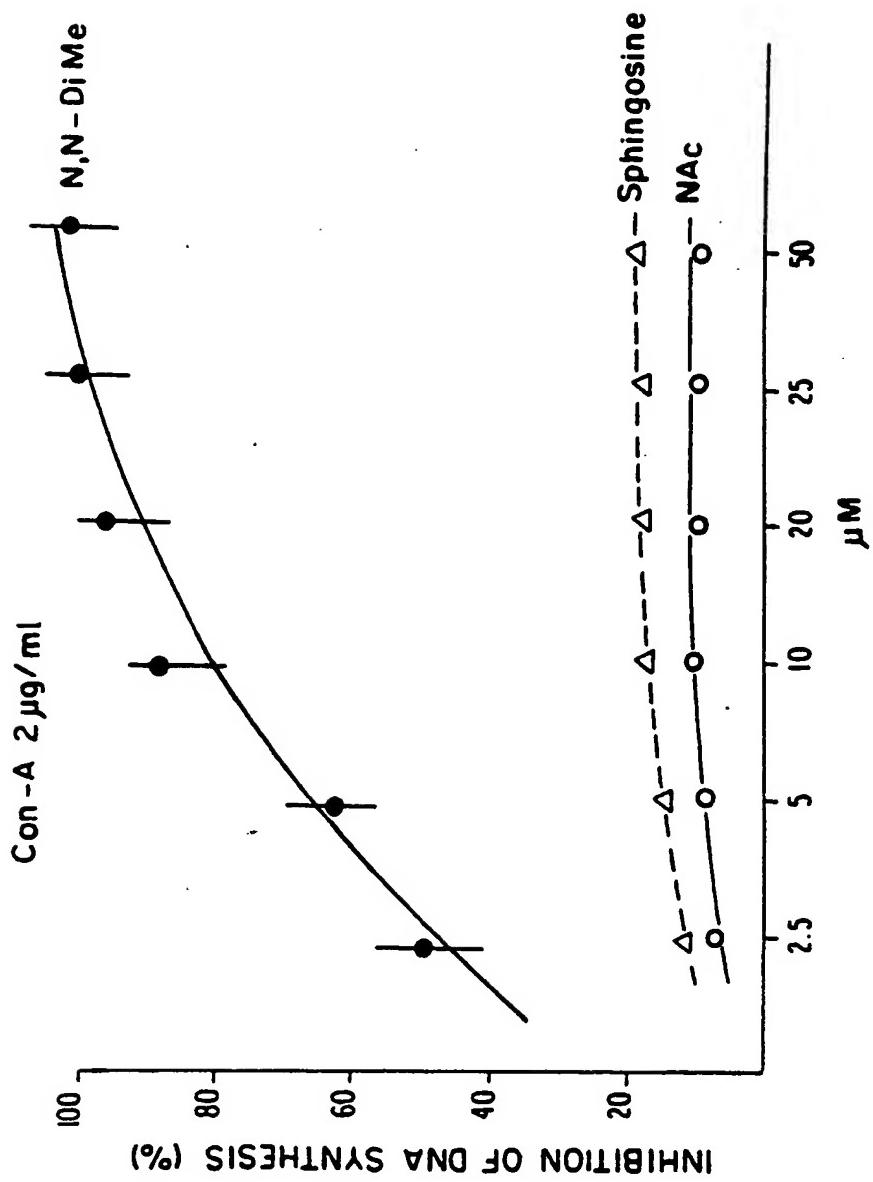


FIG. 1

